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Award Number: W81XWH-04-1-0039

TITLE: EGR-1 Functional Activation, Signaling, and Radiation Response in Prostate Cancer

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REPORT DATE: April 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Annual Summary (1 Apr 2004 - 31 Mar 2005)

4. TITLE AND SUBTITLE
EGR-1 Functional Activation, Signaling, and Radiation Response in Prostate Cancer

5. FUNDING NUMBERS
W81XWH-04-1-0039

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

In the grant proposal, we hypothesized that in prostate tumors, radiation will up-regulate pro-survival genes and confer an "induced radiation resistance" phenotype. Overexpression of EGR-1 protein will inhibit radiation-induced pro-survival genes that lead to enhanced radiation-induced apoptosis and tumor regression. Two specific aims were proposed: *Aim-1:* To determine the functional role of EGR-1 overexpression in the inhibition of radiation-induced pro-survival factors such as NFκB and Bcl-2, and its' impact on radiation response, as judged by tumor regression in prostate cancer cell lines xenografts. *Aim-2:* To determine the relative effect of radiation on the regression of prostate carcinoma *in-situ* in TRAMP mice versus TRAMP/Egr-$1^{-/-}$ mice. After Dr Dimova's resignation (prior PI), I was selected to be the PI of this grant. The following tasks were achieved: Aim 1: Two trials were conducted to assess the impact of Adenoviral EGR-1 therapy in combination with radiation. In Trial 2, tumor tissues were collected for molecular signaling analysis. Aim 2: The breeding of EGR-1 knockout mice and TRAMP mice is in progress. We are currently increasing the population of double transgene TRAMP/EGR-1-/- for further experiments. Imaging protocol has been developed and regression of prostate tumor in-situ was assessed in few animals.

14. SUBJECT TERMS
Early growth response-1, prostate cancer, apoptosis, radiation

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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I. INTRODUCTION

Carcinoma of the prostate, the most frequently diagnosed cancer in American males, continues to show a steady increase in the annual incidence of newly diagnosed cases. This tumor is characterized by a remarkably variable, often prolonged natural history. Five-year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The molecular basis of these histologic changes and clinical outcome is not known. Analysis of molecular indicators of cell proliferation or programmed cell death (apoptosis) will be of value in identifying patient subsets that may require possible radiation dose escalation in order to achieve complete local control of prostate adenocarcinoma. In this proposal, we selected *Egr-1* gene as a molecular indicator to understand the radiation-inducible signal transduction pathway leading either to growth arrest or cell death in prostate cancer cells. We specifically chose *Egr*-1 because of the following reasons : (A) In normal prostate, upon orchiectomy (castration) *Egr*-1 was rapidly induced and this led to apoptosis of androgen-dependent cells [5,11]; (B) *Egr*-1 gene has a radiation inducible promoter and; (C) EGR-1 protein is a strong transcription activator of key genes involved in cell death pathway. To support the above facts, it has been documented that cell injury caused by cellular response to radiation leads to transient induction of *Egr-1* within 0.5 to 3 hours of exposure to X-rays in the absence of new protein synthesis [14]. Our published studies have demonstrated that *Egr*-1 (a pro-apoptotic transcription factor) is a potential inhibitor of pro-survival factors such as NFκB activity and Bcl-2 expression. *Egr*-1 was found to mediate radiation-induced pro-apoptotic action via TNF-a [4] or Bax expression [10] or p53/Rb regulation [9] (reviewed in [2]). In particular, *Egr*-1 was found to inhibit NFkB activity and directly upregulate Bax in mutant p53 cell background. Further, our recent data has shown that *Egr*-1 may transcriptionaly upregulate Par-4 since DU-145 cells overexpressing *Egr*-1 showed elevated levels of Par-4 mRNA and protein (a putative EGR-1 binding site is present in Par-4 promoter). Thus, these signaling events were concluded by extensive *in-vitro* studies using prostate cancer cells as well as MEF isogenic cells from Egr-1 knock-out mice [9,10]. Together, these observations strongly suggest that *Egr-1* induction is involved in radiation-inducible signal transduction.

External beam and brachytherapy are the most common treatment modality for the treatment of prostate cancer. Because 50 to 60% of prostate cancer patients have histologically positive biopsies after radiation therapy with an increased risk of local recurrence, distant metastases and death, the status of molecular events in these positive histological biopsies needs to be elucidated. One such molecular events include the tumor suppressor gene, p53, which is a potent transcriptional repressor of Bcl-2 [19], whereas, NFkB is potent transcriptional activator of Bcl-2 [12,22]. On cellular insults, p53 represses Bcl-2 [19] and simultaneously Bax is induced by p53 [20] causing a change in the bcl-2: bax ratio leading to the culmination of downstream cell-death processes. On the contrary, NFkB mediated induction of Bcl-2 protein will lower the Bax ratio leading to enhanced cell survival. Many studies have shown that ionizing radiation decreases the Bcl-2 protein levels in p53 wild-type cell lines causing enhanced cell death [6,23]. Whereas, in cell lines lacking wild-type p53 protein function, radiation was found to upregulate NFkB activity and Bcl-2 expression [16]. In prostate cancer, loss of p53 function and radiation-induced NFkB activity and Bcl-2 upregulation might together contribute towards enhanced resistance to apoptosis [7,16]. Thus, through this signaling, the prostate tumors acquire "induced radiation resistance" phenotype. Hence, it is imperative to identify other pro-apoptotic genes that can function via a p53-independent mechanism to regulate NFkB activity and Bcl-2 expression and further to design novel approaches to mitigate the expression of such anti-apoptotic genes and eliminate the "induced radiation resistant phenotype" in prostate tumors. This "induce radiation resistance" phenotype is commonly prevalent in prostate tumors as demonstrated by our previous findings that aberrant expression of EGR-1 protein in mutant form was found to be a potential marker of radio-resistance in prostate cancer [3]. Based on the aforementioned reasoning, it is hypothesized that in prostate tumors harboring dysfunctional EGR-1 protein, radiation will up-regulate pro-survival genes and confer an "induced radiation resistant phenotype". Overexpression or restoration of EGR-1 protein function will inhibit radiation-induced pro-survival genes that will lead to enhanced radiation-induced apoptosis and tumor regression. Thus, restoration of EGR-1 function coupled with radiation will physiologically change the phenotype from "induced radiation resistance" to "hyper-radiation sensitivity". Together, these observations underscore the need to formally determine the physiological significance of Egr-1 function in radiation treated prostate cancer cells using *in-vivo* prostate cancer models.

To ascertain the feasibility of experiments required to address the questions proposed in our specific aims, we conducted these studies to test the hypothesis we had proposed in this grant application. In particular, we developed three methodologies that will be used in the course of our proposed study. The first methodology we had standardized is acquisition of mouse prostate images by magnetic resonance imaging (MRI). The second methodology is standardization of electro-mobility shift assay (EMSA, gel-shift) to determine the DNA binding activity of EGR-1 protein in tumor specimens obtained from prostate cancer patients. And the third methodology is standardization of transfection using EBS-CAT reporter plasmid in primary cultures of prostate cancer xenografts. These results of our study may provide valuable clues that the loss of *Egr*-1 gene function will result in enhanced tumor radio-resistance.

II. BODY

<u>Specific Aim-1:</u> **To determine the functional role of EGR-1 overexpression in the inhibition of radiation-induced pro-survival factors such as NFκB and Bcl-2, and its' impact on radiation response, as judged by tumor regression, in nude mice xenografts developed by prostate cancer cell lines. EGR-1 overexpression will be achieved using adenoviral constructs such as Ad/GFP-EGR-1 and Ad/GFP.**

Our previous studies have demonstrated in various prostate cancer cell lines and as well as isogenic cell system that EGR-1 protein is a pro-apoptotic sensitizer of ionizing radiation [4,8-10]. The purpose of this study was to evaluate the combination effect of radiation therapy and EGR-1 adenovirus gene therapy on the growth of prostate tumors in nude mouse model. These tasks were studied using PC-3 p53 null prostate tumor cells injected into nude mice. Tumor growth combined with radiation treatments and adenoviral injections of EGR-1 and subsequent evaluation of tumor volume was performed to assess the influence of EGR-1 expression in radiation-induced tumor growth regression. Nude mice (Nu/Nu) aged 4-6 weeks, were injected with 100 μl 5×10^6 PC3 cells subcutaneously on the medial side of the thigh, just above the level of the stifle. The animals were observed for 3-4 weeks until the xenografts reached tumor volume of 0.5 cm^3. In Trial I and II, a total of 55 animals were randomized into 6 groups for the following treatment regimen: Group I: parental xenografts only with no treatments; Group II: parental xenografts treated with total of 20 Gy irradiation; Group III: Ad/GFP injected xenografts only; Group IV: Ad/GFP-EGR-1 injected xenografts only; Group V: Ad/GFP injected xenografts treated with total of 20 Gy irradiation; and Group VI: Ad/GFP-EGR-1 injected xenografts treated with total of 20 Gy irradiation. Ad/GFP or Ad/GFP-EGR-1 constructs were injected into the tumor at a dose of 10 μl equivalent to 100 MOI (3×10^8 pfu) at the first day of treatment, followed by 5 days of gamma irradiation with 2 Gy dose per day using a ^{137} Cesium source with the whole body of the animal shielded by a 5 cm thick cerroband block and only the side of the thigh with the tumor was exposed. Following the next two days after 5 days of radiation, the animals received two more injections with 10 μl (100 MOI) of Ad/GFP or Ad/GFP-EGR-1 constructs into the tumor, which were subsequently treated with 2 Gy dose of gamma irradiation for remaining 5 days. On the 14th day of treatment, the animals were injected one last time with 10 μl of 100 MOI Ad/GFP or Ad/GFP-EGR-1 constructs into the tumor and were observed for the following two weeks. Mice were euthanized on the 26th day of treatment for tissue harvest. Tumor volume assessment was done and plotted into a graph to compare different treatments. No differences were observed between Group I and III, suggesting that Ad/GFP alone did not exert any tumor regression effect. However, when compared to Group IV and I, Group II and Group V showed modest regression but was not significant suggesting that Ad/GFP-EGR-1 alone did not confer tumor regression. Interestingly, when Ad/GFP-EGR-1 was combined with radiation, an initial significant enhancement of tumor regression was observed followed by prolonged tumor growth delay ($p<0.0001$) (Figure 1). The survival rates of the animals treated with Ad/GFP-EGR-1 plus 20 Gy irradiation were 100% at day 37 from the onset of treatment compared to the other treatment groups. Histological analysis of tumors excised at the termination of the treatment revealed a significant increase in number of apoptotic cells as a result of dynamic changes in EGR-1 pro-apoptotic target genes. One major concern from this observation was that the Ad/EGR-1 gene therapy promoted tumor growth when compared to Ad/GFP alone. It is known that Egr-1 regulates the expression of TGF-β protein [18]. Recently, it was demonstrated that in metastatic PC-3 cells, exposure to exogenous TGF- β causes increased cell adhesion [15]. This increase cell adhesion may have led to an increase tumorogenic growth in the PC-3 xenografts treated with

Ad/GFP-EGR-1. Altogether, these results imply similar results as observed in *in-vitro* studies, strongly suggesting that Egr-1 is pro-apoptotic sensitizer of radiation in prostate cancer.

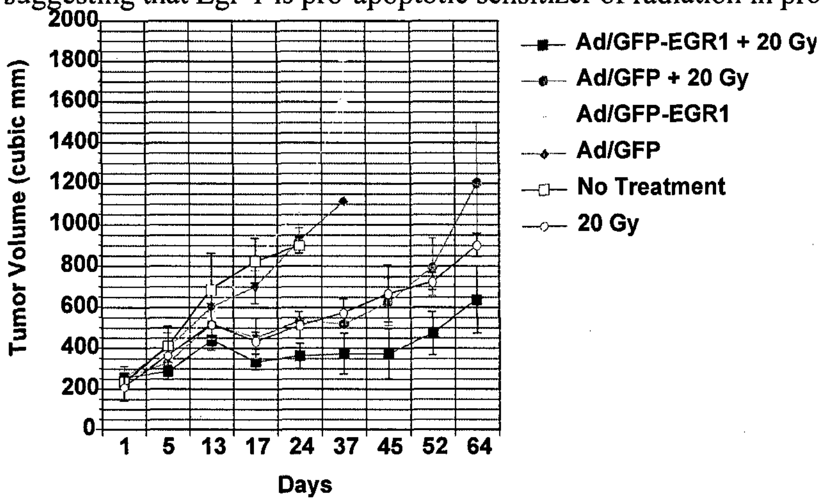

Figure 1. Graph showing regression of PC-3 xenografts in nude mice treated with radiation and adenoviral EGR-1 therapy.

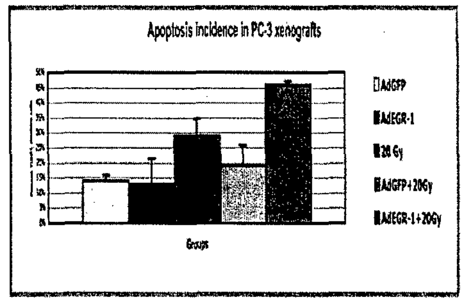

Figure 2. Bar graph showing the incidence of in-situ tumor apoptosis for Group II to VI. The untreated group I apoptotic index levels were normalized to the treated groups II to VI. Error bars represent standard deviation obtained from a mean of two experiments.

Our previous in vitro study showed that adenoviral overexpression of Egr-1 potentiates radiation-induced cell death [2]. To understand the mode regression in the PC-3 xenografts, tumor specimens were harvested in each treatment group three after the last treatment. The percent of TUNEL positive cells obtained from all groups was normalized to the parental group received no treatment (Figure 2). No difference in apoptosis initiation was observed in Group III and IV suggesting that adenovirus therapy alone does not causes enhancement of cell death. However, when compared to Group II and VI obvious increase of apoptotic incidence with maximal percentage of apoptotic cells in Group VI was demonstrated. There was a modest difference in percentage of TUNEL positive cells between group II and V. These results correlate with regression of prostate tumor xenografts in the xenografts treated with combination of ionizing radiation and adenovirus.

Western blot analysis was performed using total protein lysates from the untreated and treated tumor tissues to assess the gene signaling cascades induced by radiation plus adenoviral gene therapy. The probes used in this study were Egr-1, Bcl-2, p21 and β-actin (data not shown because it is currently under analysis).

<u>Specific Aim-2:</u> To determine the relative effect of radiation on the regression of prostate carcinoma *in-situ* in TRAMP mice versus TRAMP/Egr-1$^{-/-}$ mice.

Tumor xenograft model is one of long-standing pre-clinical screening model to set the stage for clinical trials. However, they do not closely mimic to *in-situ* tumor situation. In-situ tumor model such as "Transgenic Adenocarcinoma of Mouse Prostate (TRAMP)" has been extensively used in understanding the prostate tumor biology [13]. Further, Milbrandt's group from Washington University at St Louis developed the Egr-1 knock-out mice [17]. Recently, it was shown that cross-breeding of Egr-1$^{-/-}$ mice with transgenic mouse models of prostate cancer such as CR2-T-Ag and TRAMP mice caused a significant delay from PIN to neoplasia of prostate [1]. Once the prostate tumor was developed, no differences in the tumor growth were observed. Using a similar cross-bred model between Egr-1$^{-/-}$ and TRAMP, we propose to study the functional role of Egr-1 in regulating radiation sensitivity in an in-situ tumor situation since in humans, the prostate tumors lack EGR-1 function [3,21]. To perform these experiments, the transgenic TRAMP mice breeders and Egr-1 knock-out mice breeders were obtained from Dr Greenberg and Dr Milbrandt respectively. These are being cross-bred to obtain sizable Egr-1$^{-/-}$/TRAMP population. Currently through this mode breeding we have TRAMP -/-, Egr-1 +/- (n=2); TRAMP -/-, Egr-1 +/+ (n=3); TRAMP +/-, Egr-1 +/+ (n=9); TRAMP +/-, Egr-1 +/- (n=10); TRAMP +/-, Egr-1 +/+ (n=2); TRAMP +/+, Egr-1 +/- (n=4); TRAMP +/+, Egr-1 +/+ (n=6). The cross-breeding data are given in Table 1.

Table 1. Mouse cross-bread TRAMP/Egr-1 population.

DOB	gender	genetic profile
5/7/2003	F	TRAMP +/-,EGR +/-
5/8/2003	F	TRAMP +/-, EGR +/-
8/8/2003	F	TRAMP -/-, EGR +/+
8/9/2003	F	TRAMP -/-, EGR +/+
10/22/2003	F	TRAMP +/-, EGR +/-
10/15/2003	F	TRAMP +/-, EGR +/+
10/16/2003	F	TRAMP -/-, EGR +/+
10/17/2003	F	TRAMP +/+, EGR +/+
8/21/2003	M	TRAMP +/-, EGR +/-
8/22/2003	M	TRAMP -/-, EGR +/-
9/23/2004	M	TRAMP -/-, EGR +/-
9/23/2004	M	TRAMP +/+, EGR +/+
8/27/2003	M	TRAMP +/-, EGR +/-
6/14/2004	F	TRAMP +/-, EGR +/-
6/14/2004	F	TRAMP +/+, EGR +/+
9/23/2004	F	TRAMP +/+, EGR +/+
9/23/2004	F	TRAMP +/+, EGR +/+
6/14/2004	M	TRAMP +/- EGR +/+
6/15/2004	M	TRAMP +/+, EGR +/+
6/16/2004	M	TRAMP +/-, EGR +/+
6/17/2004	M	TRAMP +/-, EGR +/-
6/14/2004	F	TRAMP +/+, EGR +/-
6/15/2004	F	TRAMP +/+, EGR +/-
5/27/2004	F	TRAMP +/+, EGR +/-
5/28/2004	F	TRAMP +/+, EGR +/-
5/29/2004	F	TRAMP +/- EGR +/+
9/24/2004	M	TRAMP +/- EGR +/+
9/25/2004	M	TRAMP +/- EGR +/+
9/26/2004	M	TRAMP +/- EGR +/+
9/27/2004	F	TRAMP +/-, EGR +/-
10/17/2004	F	TRAMP +/-, EGR +/-
10/172004	F	TRAMP +/-, EGR +/-
10/17/2004	M	TRAMP +/- EGR +/+
10/17/2004	M	TRAMP +/- EGR +/+
10/17/2004	M	TRAMP +/- EGR +/+
10/17/2004	M	TRAMP +/- EGR +/+

Further, to monitor in-situ tumor regression and tumor growth delay in TRAMP mice, we developed a magnetic resonance imaging technique to acquire prostate images. These prostate images will be used for volumetric measurements and treatment planning for external beam radiation. Thus, we plan to irradiate in-situ prostate tumors and monitor the tumor regression pattern in Egr-1 positive and negative TRAMP tumors. Data pertaining to acquisition protocol of mouse prostate images by MRI is given below. Images were acquired on mice using a 1.5T Siemens Vision imager. The animals were anesthetized with ketamine and placed in a Plexiglas holder so that the lower quarters of the animal were placed inside a 2.5 cm diameter surface coil held vertically. MR visible fiducials were placed on the belly of the animal to aid in the treatment planning. The animal was held in place by clamping its legs in appropriately machined grooves in the Plexiglass holder but other wise no further restraint was used. After localizer images identified the region of interest a high-resolution three dimensional, spoiled gradient echo image series was acquired to visualize the location and dimensions of the tumor. The images had acquisition parameters of TR/TE/α= 25 ms/11 ms/ 30°. The image dimensions were 80x80x40 mm and were acquired with a 256x256x80 matrix to produce images with an inplane resolution of

0.3125x0.3125x 0.500 mm. Before imaging the animal, Gd-DTPA (0.1 mmol/kg) was injected into the tail vein. The contrast agent accumulated in the bladder and permitted easier identification of the bladder. Images were reformed to sagittal sections using software on the imager and sent via the hospital's PACS network to the radiation therapy treatment planning computers. To better visualize the tumor we also acquired diffusion weighted images with diffusion weighting of b=0 and 222 mm^2/sec. From these images apparent diffusion coefficient (ADC) images were calculated on a pixel-by-pixel basis (Figure 3). <u>Tumor volume characterization:</u>

MR images of the mouse prostate were downloaded to a commercial 3D treatment planning system (CMS FOCUS, Computerized Medical Systems, St. Louis MO) for volumetric and depth analysis. The downloaded images consisted of coronal views of the prostate region. Contours of the identified normal prostate was done by Dr. Rowland. Volume information was then extracted utilizing the Dose Volume Histogram (DVH) utilities provided in FOCUS (Figure 4). We have confirmed with MR imaging prostate tumors in 3 male TRAMP mice. The mice were treated with single beam radiation. The radiotherapy schedule consists of a total 20 Gy dose of radiation and was given in 2 Gy fraction per day for 10 days. MR images were taken one week after last dose irradiation and tumor volumes were calculated from MR images and plotted into a graph for comparison with the volume before treatment. TRAMP 7 was imaged at 28 weeks of age and had a large prostatic tumor with 3.00 cc volume. This mouse died after second 2 Gy fraction due to bleeding in the tumor. TRAMP 8 was imaged at 28 weeks of age and had prostatic tumor with estimated volume of 0.55 cc. After radiotherapy, the in-situ prostate tumor regressed to a volume of 0.08 cc. TRAMP 12 was imaged at 32 weeks age and had prostatic tumor with estimated volume of 0.36 cc. After radiotherapy, the in-situ prostate tumor regressed to a volume of 0.06 cc (Figure 5). These images, tumor volume measurements and radiation treatment planning validate that we have put together an expert team to achieve the objectives proposed in the aim 2.

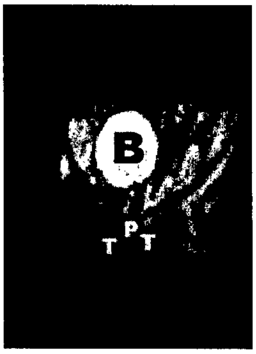

Figure 3. Coronal image of mouse normal prostate acquired by MRI showing bladder (B), prostate (P) and testis (T). The distorted image above is caused due to respiratory motion in mouse under anesthesia.

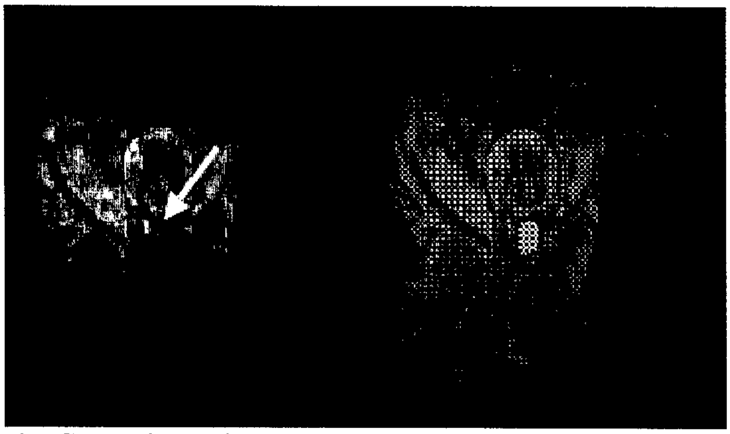

Figure 4. Coronal MR images of the abdomen of a normal mouse. The arrow in the image points to the prostate just caudal to the bladder. Image on the right shows the user-defined overlay on the prostate to estimate the prostate volume.

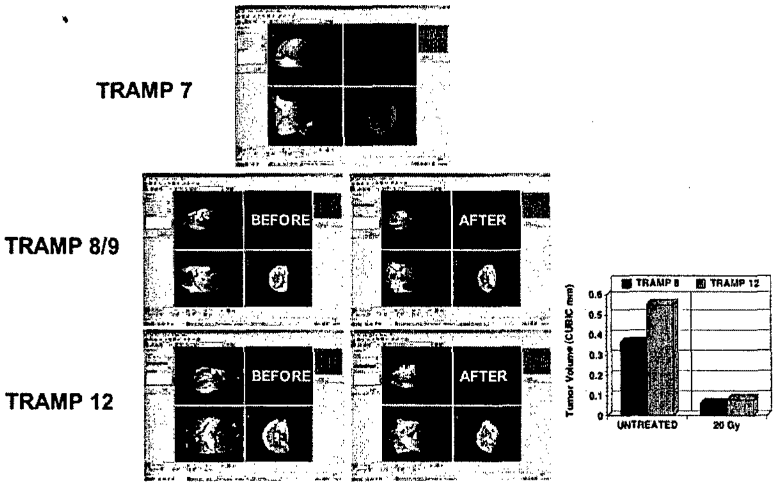

Figure 5. Prostate in-situ tumor volume before and after radiation therapy. TRAMP 7 died during radiation therapy. The tumor volume is marked in red.

EGR-1 DNA binding and EBS-CAT reporter activity in xenograft prostate tumors. We recently reported that prostate tumors overexpress EGR-1 protein in mutant form and correlates with treatment failure [3]. Because of mutation, the EGR-1 protein might lack transactivation function and this loss of function may lead to accumulation of EGR-1 protein. This is supported by a recent study in which the function of EGR-1 in benign and tumor regions of prostate is due to the difference in the association of EGR-1 with microtubules, which is exclusive to the benign cells of the prostate and is requisite for the nuclear translocation and transcriptional activity of EGR-1 [21]. Further, this study demonstrated that the differences in EGR-1 expression between benign and malignant prostate cells extend beyond cellular levels, which was confirmed by immunohistochemistry in human tissues (obtained as a control from the University of Kentucky tissue bank, since this study does not involve the use of human specimens). By gel-shift analysis using a 2x EGR-1 binding site oligo probe, we found that neither the benign tissue nor the tumor tissue of prostate showed EGR-1 DNA binding activity (Figure 6). However, by immunohistochemistry, EGR-1 protein was significantly overexpressed in the control human tumor and was absent in the control benign tissue. These results indicate that lack of basal level of EGR-1 protein in benign prostate may attribute to lack of EGR-1 DNA binding activity. However, in xenograft tumor tissue, EGR-1 was significantly overexpressed by adenoviral EGR-1 injection and EGR-1 DNA binding activity was present suggesting that the overexpressed EGR-1 protein in xenograft tumor harbors DNA binding function. This finding may not necessarily infer on the EGR-1 transactivation function. To further ascertain, we established a methodology to assess the endogenous EGR-1 function using 3xEBS-CAT reporter transient transfection system combined with ionizing radiation to induce the endogenous EGR-1 transactivation function. This was tested in PC-3 tumor xenograft tissue and primary cultures of normal and tumor of the prostate. The PC-3 xenograft tumors infected with Ad/GFP or Ad/EGR1-GFP and 24 hours later primary cultures from these tumor xenografts were established in RPMI supplemented with 3% fetal calf serum. After 48 hours, these cultures were transiently transfected and irradiated after 24 hours and cells were lysed using freeze-thaw technique to assess the CAT activity. The results in a figure form are not shown here due to weak autoradiography results. However, the weak autoradiography findings suggest that adenoviral EGR-1 infected and / or irradiated tumor primary cultures showed increased acetylation conversion than the non-irradiated or adenoviral GFP infected primary cultures of PC-3 xenografts.

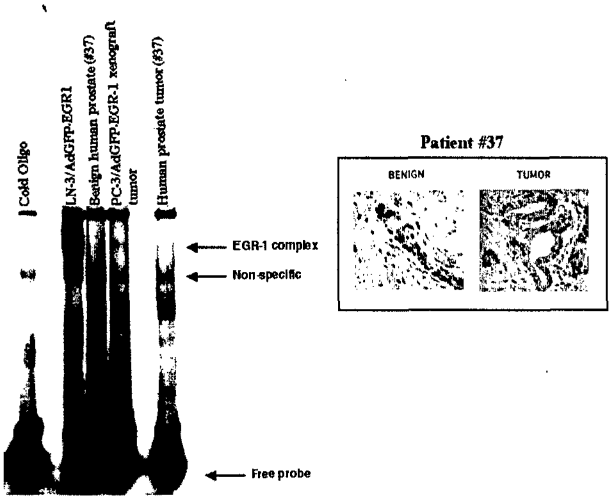

Figure 6. EGR-1 DNA binding activity PC-3 xenografts infected with Ad/GFP-EGR-1 show EGR-1 DNA binding activity. The controls benign and tumor tissue of prostate cancer patients show lack of EGR-1 DNA binding activity.

III. KEY RESEARCH ACCOMPLISHMENTS

1. Adenoviral EGR-1 gene expression is a potent sensitizer of radiation in regressing prostate cancer xenografts in nude mice.
2. Radiation sensitization by adenoviral EGR-1 gene therapy was found to be associated with enhanced in-situ tumor apoptosis.
3. Established EMSA and reporter CAT activity protocol to assess the EGR-1 transactivation function in-situ tumors infected with Ad/EGR-1 or Ad/EGR-1 plus radiation therapy (method accomplishment).
4. MRI based imaging of mouse prostate was established (Imaging Protocol accomplishment)
5. Established external beam radiation treatment planning protocol to irradiate mouse prostate tumors.

IV. REPORTABLE OUTCOMES

Since the granting period, we have presented the results in two conferences and we are in progress for completing a manuscript for publication. These reportable outcomes were pertaining to the findings of specific aim 1. The details of the presentation and manuscript are given below:

Presentation

1. Marianna Sultanov, Neviana G. Dimova and Mansoor M. Ahmed. Adenoviral overexpression of EGR-1 enhances the effects of ionizing radiation on prostate cancer xenografts. 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida. March 2004.

2. Dimova, N. and Ahmed, M.M. Adenoviral overexpression of EGR-1 enhances the effects of ionizing radiation on prostate cancer xenografts. Presented at Gordon Research Conference on "Radiation Oncology" (January 2003) at Ventura, CA.

Manuscript in preparation

Sultanov, M. and **Ahmed, M.M.** Adenoviral overexpression of EGR-1 enhances the effects of ionizing radiation on prostate cancer xenografts. (In preparation).

V. CONCLUSIONS

The major conclusion of specific aim 1 demonstrated that EGR-1 in combination with radiation significantly regressed the PC-3 tumor cell line xenografts. These findings show similar results as our previously reported *in-vitro* studies, strongly suggesting that Egr-1 is a pro-apoptotic sensitizer of radiation in prostate cancer. Studies are in progressing to understand the gene signaling mechanisms underlying the radio-sensitizing effects of adenoviral gene therapy.

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VII. APPENDICES

Copies of the abstracts and posters presented at Gordon Research Conference on Radiation Oncology and American Association for Cancer Research.

ADENOVIRAL OVEREXPRESSION OF EGR-1 ENHANCES THE EFFECTS OF IONIZING RADIATION ON PROSTATE CANCER XENOGRAFTS

Neviana Dimova and Mansoor M Ahmed
Department of Radiation Medicine, University of Kentucky, Lexington, KY.

The purpose of this study was to evaluate the combination effect of radiation therapy and EGR-1 adenovirus therapy on the growth of prostate tumors in nude mouse model. These tasks were studied using PC-3 p53 null prostate tumor cells injected into nude mice. Tumor growth combined with radiation treatments and adenoviral injections of EGR-1 and subsequent evaluation of tumor volume were performed to assess the influence of EGR-1 expression in radiation-induced tumor growth regression. Nude mice (Nu/Nu) aged 4-6 weeks, were injected with 100 μl $5x10^6$ PC3 cells subcutaneously on the medial side of the thigh, just above the level of the stifle. The animals were observed for 3-4 weeks until the xenografts reached tumor volume of 0.5 cm^3. At this time, a total of 25 animals were randomized into 5 groups for the following treatment regimen: Group I: Ad/GFP-EGR-1 injected xenografts treated with total of 20 Gy irradiation; Group II: Ad/GFP injected xenografts treated with total of 20 Gy irradiation ; Group III: Ad/GFP-EGR-1 injected xenografts only; Group IV: Ad/GFP injected xenografts only and ; Group V: parental xenografts only with no treatments. Ad/GFP or Ad/GFP-EGR-1 constructs were injected into the tumor at a dose of 10 μl equivalent to 100 MOI ($3x10^8$ pfu) at the first day of treatment, followed by 5 days of gamma irradiation with 2 Gy dose per day using a ^{137} Cesium source with the whole body of the animal shielded by a 5 cm thick cerroband block and only the side of the thigh with the tumor was exposed. Following the next two days after 5 days of radiation, the animals received two more injections with 10 μl (100 MOI) of Ad/GFP or Ad/GFP-EGR-1 constructs into the tumor, which were subsequently treated with 2 Gy dose of gamma irradiation for remaining 5 days. On the 14^{th} day of treatment, the animals were injected one last time with 10 μl of 100 MOI Ad/GFP or Ad/GFP-EGR-1 constructs into the tumor and were observed for the following two weeks. Mice were euthanized on the 26^{th} day of treatment for tissue harvest. Tumor volume assessment was done and plotted into a graph to compare different treatments. No differences were observed between Group V and IV, suggesting that Ad/GFP alone did not exert any tumor regression effect. However, when compared to Group IV and V, Group II and Group III showed modest regression but was not significant suggesting that radiation or Ad/GFP-EGR-1 alone confers tumor regression. Interestingly, when Ad/GFP-EGR-1 was combined with radiation, a significant enhancement in tumor regression was observed till 26 day ($p<0.0001$). Thus, the results of this study demonstrated that EGR-1 in combination with radiation significantly regressed the PC-3 xenografts. These findings imply similar results as observed in our reported *in-vitro* studies, strongly suggesting that Egr-1 is pro-apoptotic sensitizer of radiation in prostate cancer.

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ABSTRACT

The purpose of this study was to evaluate the effect of radiation in combination EGR-1 adenovirus therapy on the growth of prostate tumors in the nude mouse model using PC-3, a p53 null prostate tumor cell line. Six groups were designed for the following treatment regimen: Group I: parental xenografts only with no treatments; Group II: parental xenografts only with total dose of 20 Gy irradiation; Group III: Ad/GFP injected xenografts only; Group IV: Ad/GFP-EGR-1 injected xenografts only; Group V: Ad/GFP injected xenografts treated with total dose of 20 Gy irradiation; and Group VI: Ad/GFP-EGR-1 injected xenografts treated with total dose of 20 Gy irradiation. Ad/GFP or Ad/GFP-EGR-1 constructs were injected into the tumor at a dose of 100 MOI ($3x10^8$ PFU) at the first day of treatment, followed by 2 Gy radiation dose per day to the tumor for 5 days. On 7th and 8th days, the animals received two more injections of 100 MOI of Ad/GFP or Ad/GFP-EGR-1 adenoviral constructs into the tumor, and they were subsequently treated with 2 Gy dose of radiation per day for 5 days. On the 14th day of treatment, the animals were injected with a final intra-tumoral dose of 100 MOI of Ad/GFP or Ad/GFP-EGR-1 adenovirus and were observed for the following 4 weeks. No differences were observed between Group I and III, suggesting that Ad/GFP alone did not exert any tumor regression effect. However, when compared to Group IV and I, Group II and Group V showed modest regression but was not significant suggesting that Ad/GFP-EGR-1 alone did not confer tumor regression. Interestingly, when Ad/GFP-EGR-1 was combined with radiation, an initial significant enhancement of tumor regression was observed followed by long tumor growth delay ($p<0.0001$). Thus, the results of this study demonstrated that EGR-1 in combination with radiation significantly regressed the PC-3 xenografts. These findings show similar results as our previously reported *in-vitro* studies, strongly suggesting that Egr-1 is a pro-apoptotic sensitizer of radiation in prostate cancer.

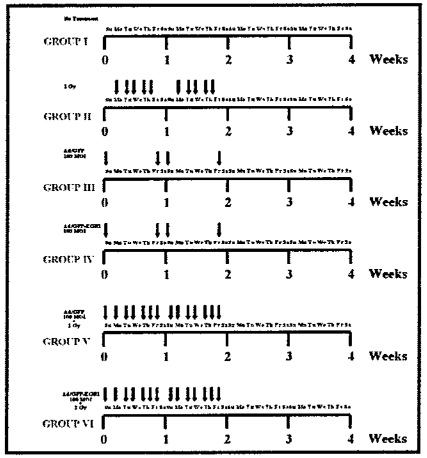
BACKGROUND

Tumor aggressiveness correlates with enhanced resistance to apoptosis, which tumors often acquire by mutations and over expression of cellular genes that signal the pro-apoptotic functions. The apoptotic pathway consists of an early component that includes molecular events specific for an inducer or a group of inducers and of downstream effector components common to diverse apoptotic signals. Apoptosis has also been reported in a variety of experimental tumor systems following exposure to radiation. Ionizing radiation alters the expression of specific genes, the products of which may contribute to the events leading to apoptotic cell death. Ionizing radiation exposure is associated with activation of certain immediate-early genes that function as transcription factors. These include members of *jun* or *fos* and early growth response (EGR) families.

The EGR gene family includes EGR-1, EGR-2 (Krox-24), EGR-3, NGFI-C, and recently identified EGR-α, and the tumor suppressor, Wilms' tumor gene product, WT1. The *Egr* family shows high degree of homology in the amino acids constituting the zinc finger domain and binds to the same GC-rich consensus DNA sequence. The *Egr*-1 gene product, EGR-1, is a nuclear protein, which contains three zinc fingers of the C_2H_2 subtype. Structure-function mapping studies on EGR-1 protein suggest that the amino acids constituting the zinc finger motif confer DNA binding function, whereas the NH_2-terminus amino acids confer transactivation function. It is interesting to note that within this family of transcription factor, EGR-1 was found to be a positive activator of transcription, whereas WT1 is a transcriptional repressor, both acting via binding to the same GC-rich consensus sequence in reporter constructs. Depending on the cell type, EGR-1 may behave as a positive or negative regulator of gene transcription. These findings suggest that the cellular context determines the transcriptional regulatory functions of the EGR-family DNA binding proteins. The EGR-1 GC-rich consensus target sequence, has been identified in the promoter regions of: (a) transcription factors, such as MYC and NUR77; (b) growth factors or their receptors, such as TGF-β1, TNF-α, PDGF-A and PDGF-B, IGF-II, βFGF, or EGF-receptor; (c) cell cycle regulators such as the retinoblastoma susceptibility gene *Rb*, cyclin D1 and c-Ki-ras; and (d) thymidine kinase, an enzyme crucial in DNA biosynthesis.

In this study, we selected *Egr-1* gene as a molecular indicator, to understand the radiation-inducible signal transduction pathway leading either to growth arrest or cell death in prostate cancer cells. We specifically chose *Egr*-1 because: A. In normal prostate, upon orchiectomy (castration) *Egr*-1 was rapidly induced and this led to apoptosis of androgen-dependent cells; B. In most types of cells, ionizing radiation caused increased EGR-1 expression and; C. EGR-1 protein is a strong transcription activator of key genes involved in the cell death pathway. Additionally, *Egr-1* apart from being a potential transcriptional regulator, it harbors the radiation-inducible promoter. Through these distinct induction pathways, *Egr-1* has been linked to signaling events initiating cell phenotypic response to radiation injury.

EXPERIMENTAL DESIGN



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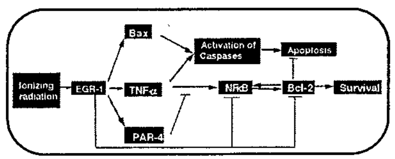
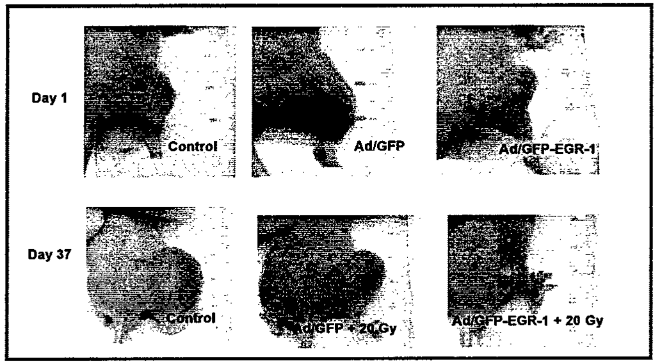
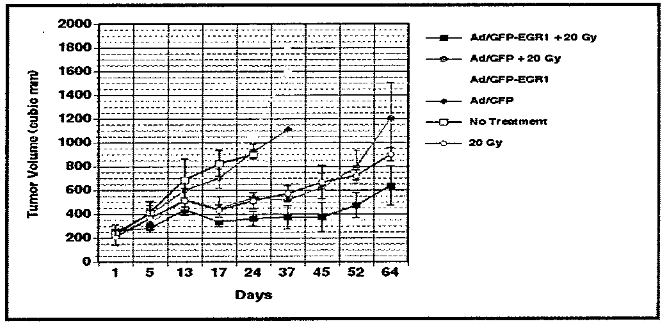


RESULTS





CONCLUSION

- The results of this study demonstrated that EGR-1 gene therapy in combination with radiation significantly regressed the PC-3 xenografts.

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#5016 Adenoviral overexpression of EGR-1 enhances the effects of ionizing radiation on prostate cancer xenografts. Neviana G. Dimova and Mansoor M. Ahmed. *University of Kentucky, Lexington, KY.*

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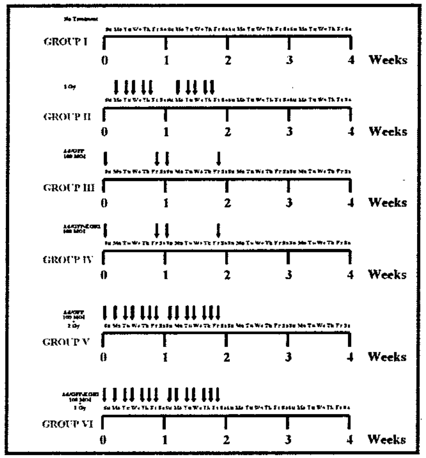

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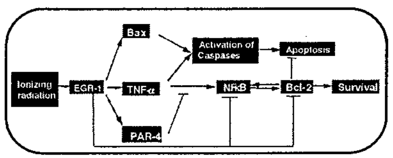

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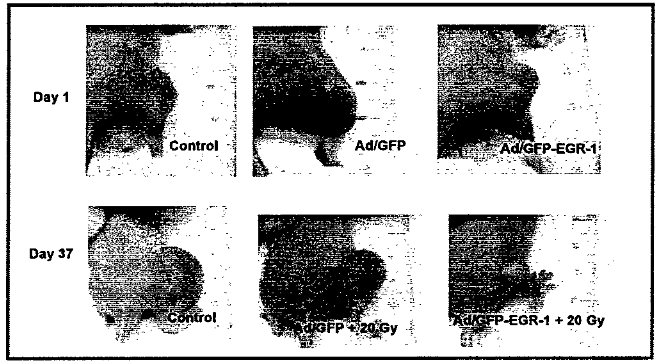

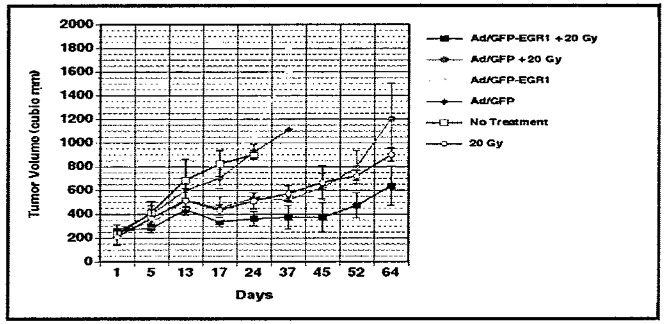

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